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An Anti-HIV Peptide Construct Derived from the Cleavage Region of the Env Precursor Acts on Env Fusogenicity through the Presence of a Functional Cleavage Sequence

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A 22-amino-acid-long multibranched peptide construct (CLV) derived from the cleavage region (KIEPLGVAPTKAKRR*VVQREKR*) of the human immunodeficiency virus (HIV) type-1 envelope precursor inhibits HIV infection (*Virology,* 1996, 223, 406–408). We attempted to characterize its activity for Env expressed *via* a recombinant vaccinia virus (rVV): gp160 cleavage was delayed, but not impaired, in the presence of CLV (10 μM), whereas neither Env production nor Env membrane expression was significantly altered. Through the synthesis of analogs, we concluded that the presence of a cleavage sequence was required for inhibition of syncytium formation by CLV in rVV-infected CD4$^+$ cell cultures: indeed, a single amino acid residue substitution (R*>S) in the cleavage sites presented by CLV abolished its activity. Other analogs allowed us to further determine the region of CLV which mediates its activity. The ability of a radiolabeled CLV analog to enter cells was also shown. Altogether, these data strongly suggest that CLV acts on Env fusogenicity at least partially through interference with gp160 processing.

Key Words: HIV Env, gp160 cleavage, Env activity, anti-HIV peptides, protein processing.

INTRODUCTION

The surface glycoprotein gp120 and the transmembrane glycoprotein gp41 are cleavage products of the gp160 precursor and the components of the mature human immunodeficiency virus (HIV) type-1 envelope (Env) (Einfeld, 1996). gp120 and gp41 are non-covalently associated at the virus surface, where they mediate HIV binding to, and membrane fusion with, CD4$^+$ cells, respectively (Moore *et al.,* 1993; Einfeld, 1996).

The cleavage of gp160 into gp41 and gp120 is required for activation of the HIV envelope fusion function: mutation of gp160 cleavage sites impairs membrane fusion through precursor cleavage inhibition (McCune *et al.,* 1988; Freed *et al.,* 1989; Bosch and Pawlita, 1990). During its biosynthetic pathway, gp160 cleavage results—likely in a trans Golgi compartment (Pfeiffer *et al.,* 1997)—from the action of proteases that have not been unequivocally identified: for some, furin activity is considered the candidate for Env cleavage but several works showed that subtilisin-like protein convertases also fulfill the expected criteria (Hallenberger *et al.,* 1992, 1997; Morikawa *et al.,* 1993; Onishi *et al.,* 1994; Decroly *et al.,* 1994, 1996, 1997; Gu *et al.,* 1995; Inocencio *et al.,* 1997; Einfeld, 1996).

Two potential conserved tryptic-like endoproteolytic cleavage sites represented by clusters of basic amino acid residues (aa) exist on gp160 (Bosch and Pawlita, 1990; Einfeld, 1996). A first site is defined by the sequence KAKRR (aa 505–509) and the other by the sequence REKR (aa 513–516). *In vitro,* furin cleaves more efficiently the REKR site when compared to the KAKRR sequence (Brakch *et al.,* 1995). About 15% of mature recombinant and viral Env is cleaved at site 1 and does not organize in oligomers (Fenouillet and Gluckman, 1992) but it is clear that only the cleavage at site 2 leads to fusogenic gp41 species (Freed *et al.,* 1989; Bosch and Pawlita, 1990): it is likely that the gp41 resulting from cleavage at site 1 is not functional because the additional polar aa sequence alters gp41 fusion peptide properties. Surprisingly, it was shown that cleavage of Env can occur when no dibasic sequence is present at the cleavage site, which raises questions about the specificity of the protease that cleaves (Dubay *et al.,* 1995).

Various linear peptides (Callebaut *et al.,* 1996; De Rossi *et al.,* 1991; Neurath *et al.,* 1992, 1995; Slepushkin *et al.,* 1993) and synthetic multibranched peptides (SPCs) (Yahi *et al.,* 1994; Benjouad *et al.,* 1995; Barbouche *et al.*, 1998a) derived from HIV Env interfere with HIV infectivity and syncytium formation in HIV-infected CD4$^+$ cell cultures. The mode of action of these peptides remains unclear but they are supposed to interfere either with Env biosynthetic events or with post-CD4-binding events. Recently, we also reported preliminary data showing that a SPC (CLV; four branches of the KIEPLGVAPTKAKRRVVQREKR motif) derived from the sequence encompassing the cleavage region of gp160 inhibits HIV infectivity without cell toxicity (Sabatier *et al.,* 1996). Based on this observation and because it was described that peptidyl chloromethylketones that possess the RXKR motif interfere with gp160 cleavage and inhibit HIV infectivity (Hal-

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TABLE 1

Quantitation of Env Expressed in the Presence (10 μM) of either a Control SPC (C) or CLV by VV9-1-Infected BHK-21 Cells (ng/10^6 Cells) 1 or 2 Days Postinfection

	Day+1	Day+2
C		
Secreted Env	1100±200	2000±300
Cell-associated Env	275± 75	400±100
CLV		
Secreted Env	1300±300	2000±700
Cell-associated Env	275± 75	375± 75

Note. Secreted Env: Env present in cell supernatants; cell-associated Env: Env present in cell lysates; $n = 5$ experiments.

lenberger *et al.,* 1992), we hypothesized that CLV may alter the cell machinery involved in Env processing and hence membrane fusion and HIV infectivity.

Here, we studied whether inhibition by CLV of syncytium formation induced by Env depends on a reduced Env production, on interference with gp160 processing, or on an altered expression at the cell surface. Through the synthesis of various CLV analogs, we determined the region of CLV which was required for the inhibitory activity. The ability of an active CLV analog to enter cells was also studied.

RESULTS

The amount of Env production was not altered in the presence of CLV

We previously reported (Sabatier *et al.,* 1996) that a 10 μM concentration of CLV abolishes syncytium formation mediated by rVV expressing HIV Env in $CD4^+$ cell cultures. These conditions totally impair HIV infectivity, whereas the control SPC C was inactive. Of note: (i) a 10 μM concentration of CLV did not display detectable cell toxicity as assessed by proliferation assays (data not shown and Sabatier *et al.,* 1996), (ii) a 1 μM concentration of CLV inhibited by about 70% rVV-induced syncytium formation, and (iii) a control rVV lacking HIV *env* failed to induce syncytium formation (data not shown).

Because CLV may act through inhibition of Env production, we examined by dot blot—to avoid any influence of Env conformation with the labeling procedure (Fenouillet and Gluckman, 1991)—the production of Env present in lysate (gp160, gp120, and gp41) and of secreted Env (gp120) 1 or 2 days postinfection. The production was not modified by the presence of CLV (10 μM) (the set of experiments with BHK-21 cells is shown in Table 1; $n = 5$ experiments; Env samples corresponding to each condition were produced in three independent cell cultures). The data obtained by ELISA were in agreement with those obtained by dot blot assay: 2 days postinfection, irrespective of the presence or the absence of CLV, coating with D7324 detected about 300 ng of cell-associated gp120/10^6 cells and about 2 μg of secreted gp120/10^6 cells and coating with D7323 detected about 200 ng of cell-associated gp41/10^6 cells.

Membrane expression was normal in the presence of CLV

One or 2 days postinfection with rVV, CEM cells producing Env in the presence or in the absence of CLV (10 μM) were incubated with a pool of HIV^+ sera to study the kinetics of Env membrane expression (Fenouillet *et al.,* 1996, 1997): cells exhibited a similar level of Env at their surface irrespective of the presence of CLV (Fig. 1). Ab dilutions ranging from 1/50 to 1/50 000 led to similar data (not shown). Surprisingly but reproducibly ($n = 3$), the presence of CLV slightly increased labeling.

CLV delayed the cleavage of the gp160 precursor

The gp160 cleavage into gp120 and gp41 in the presence or in the absence of CLV (10 μM) was examined by pulse–chase labeling. The amount of immunopurified labeled Env was similar in all cases (Fig. 2A), in agreement with the data obtained by dot blot and ELISA. According to Fenouillet and Jones (1995) and Fenouillet *et al.* (1997), in the absence of CLV, gp120 was detected after a 30-min chase, and mature gp120—the increased MW of which reflects carbohydrate maturation (Fenouillet and Jones, 1995)—was detected after a 2-h chase. Similar data were obtained when cells were incubated with SPC C (not shown). Two hours postpulse, only a very faint band of gp160 was detected: it is likely that most of the uncleaved gp160 species was significantly degraded at that time and consequently was undetectable as a 160-kDa band after β-mercaptoethanol elution (as observed and discussed in Earl *et al.,* 1991; Fenouillet and Jones, 1995). In the presence of CLV, gp160 appeared essentially uncleaved after a 30-min chase, whereas a significant cleavage was detected 2 h postpulse.

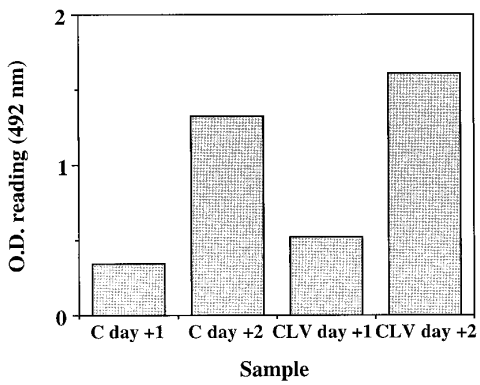

FIG. 1. Effect of CLV on Env membrane expression. One or 2 days postinfection with rVV9-1 in the presence (CLV) or in the absence (C) of CLV, cells were incubated with a pool of HIV^+ sera; staining of membrane Env was performed with peroxidase-conjugated anti-human IgG.

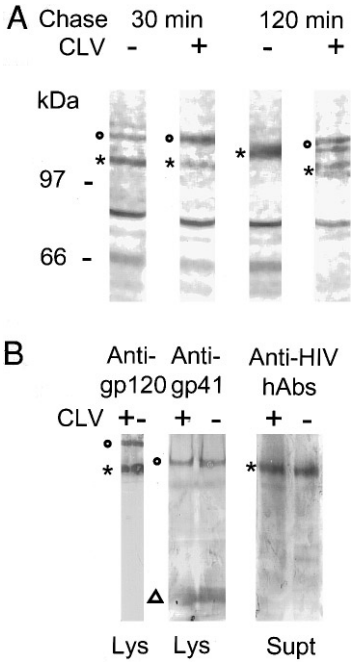

FIG. 2. Env cleavage in the presence of CLV. (A) Cells were pulse-labeled for 30 min in the presence (+) or in the absence (−) of CLV (10 μM), 18 h postinfection with rVV9-1 in the presence (+) or in the absence (−) of CLV, respectively. Cells were then chased for 30 or 120 min. After lysis of the cell pellet, Env was immunopurified using anti-HIV human Abs and analyzed by SDS–PAGE (10%) and autofluorography. MW is expressed in kDa. (B) Two days postinfection by rVV9-1 in the presence (+) or in the absence (−) of CLV (10 μM), cells were lysed (lysate: Lys) and their supernatant (Supt) was concentrated. Samples were analyzed by SDS–PAGE (10% when the staining was performed with either anti-gp41 or human anti-HIV Abs; 8% when the staining was performed with anti-gp120 Abs). (o) gp160 species; (*) gp120 species; (△) gp41 species).

High-molecular-weight gp160 was still detected at this time. The migration of the Env species as double bands has been already observed (Fenouillet *et al.,* 1997) in our discriminating SDS–PAGE/pulse–chase analysis conditions.

To further examine the ability of Env to be processed in the presence of CLV, a Western blot analysis of Env stored either in cell supernatant or in cell lysate 2 days postinfection was performed (Fig. 2B). The results supported the data presented in the pulse–chase study and those obtained by dot blot and ELISA as reported above: in the presence of CLV, a significant amount of cleaved gp41 and gp120 was detected into the cell pellet; after membrane expression, the gp120 subunit was efficiently secreted into the cell supernatant.

Thus, cleavage of Env was delayed, but not inhibited, in the presence of CLV.

CLV should present a functional cleavage site to be active

To determine the aa involved in CLV activity, we synthesized various analogs which were tested for their cell toxicity and ability to inhibit syncytium formation induced by rVV infection in $CD4^+$ cell cultures, as described in Sabatier *et al.* (1996) (Table 2). Irrespective of their sequence and length, these analogs presented a cytotoxicity similar to that of CLV. The L analog—which presents a $R_{509/516}>S$ substitution in the cleavage sites—failed to inhibit syncytium formation (Table 2). The fact that the S analog—it presents a REKR motif without aa sequence downstream this site—was inactive indicates that the REKR sequence including the R_{516} residue adjacent to the Lys core is not involved in CLV activity. In full contrast, the M analog—which contains a cleavage region with aa upstream and downstream the cleavage site—was as active as CLV, despite the deletion of a large part of the sequence upstream the first cleavage site. The ability of the various analogs to inhibit infection of C8166 cells by HIV_{MN} was also examined: according to the data presented above, the L and the S analogs were found inactive (data not shown). These data indicate that CLV exhibited its anti-HIV activity through the presence of a functional cleavage sequence on the peptide construct.

CLV Y enters CEM cells

The ability of the active CLV Y analog to enter CEM cells (Fig. 3) was studied. We observed that CLV Y was able to rapidly bind cell membrane and enter cells (the

TABLE 2

Cell Toxicity and Effect of Various SPCs Derived from the Cleavage Region of the HIV Env Precursor on Syncytium Fomation Induced in $CD4^+$ Cell Cultures by rVV Expressing HIV Env (n = 5 experiments)

		Toxicity-free concentration (M)	Syncytium formation (IC_{90})
CLV:	$[KIEPLGVAPTKAKRRVVQREKR]_4$-$K_2$-K-$\beta$A	$3{\cdot}10^{-5}$	10^{-5}
L:	$[KIEPLGVAPTKAKRSVVQREKS]_4$-$K_2$-K-$\beta$A	$3{\cdot}10^{-5}$	None
M:	$[PTKAKRRVVQREKR]_4$-K_2-K-βA	$3{\cdot}10^{-5}$	10^{-5}
S:	$[VVQREKR]_4$-K_2-K-βA	$3{\cdot}10^{-5}$	None
C:	$[GPGKTL]_8$-K_4-K_2-K-βA	10^{-4}	None

Note. $[GPGKTL]_8$-K_4-K_2-K-βA is a negative SPC control; a reduction of the syncytium score by 90% (IC_{90}) relative to the negative control was considered to result from a significant inhibitory activity of the corresponding peptide.

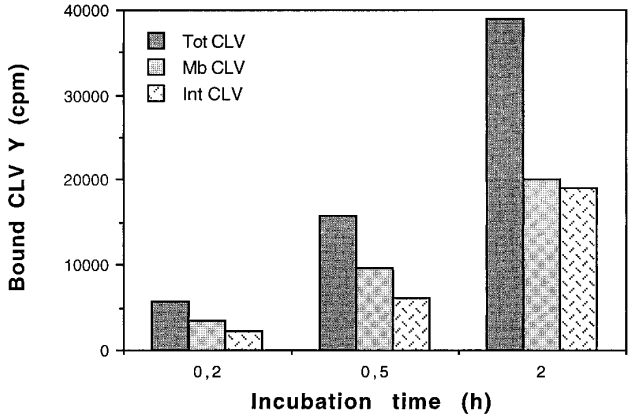

FIG. 3. Binding to, and internalization into, human $CD4^+$ lymphoid cells of a labeled active CLV analog. CEM cells were incubated at 37°C with the radiolabeled CLV Y analog. Cell-associated radioactivity when incubation was performed in the presence of NaN_3—a condition that impairs endocytosis—was considered membrane-associated CLV Y (Mb CLV). CLV Y present within the cells (Int CLV) was calculated as follows: "cell-associated radioactivity obtained when incubation was performed in the absence of NaN_3 (Tot CLV)" minus "Mb CLV."

amount of internalized CLV Y was calculated as described under Materials and Methods and in Barbouche *et al.* (1998b) as "cell-associated radioactivity obtained when incubation was performed in the absence of NaN_3" minus "cell-associated radioactivity obtained when incubation was performed in the presence of NaN_3").

DISCUSSION

This work is the third to report the design and the characterization of peptide compounds derived from the endoproteolytic cleavage sequence of HIV Env. One of these works showed that decanoylated peptides derived from the gp160 cleavage region failed to inhibit in cell culture gp160 cleavage. Moreover, their important cell toxicity did not allow further development as anti-HIV therapeutic agents (Decroly *et al.,* 1994).

In strong contrast, the pioneer work by Hallenberger *et al.* demonstrated that a set of peptide chloromethylketones (PCMK) which are derived from the gp160 cleavage region "interfere with Env cleavage and hence its activation" (Hallenberger *et al.,* 1992). It is important to note that, in this work, the authors showed that an important cleavage of Env does exist (about 50% when Env was expressed by rVV in this study) in the presence of PCMK and that despite the expression of an important amount of cleaved Env, HIV infectivity is abolished.

We present here data which confirm and expand this latter work. Indeed, we showed that a 10 μM concentration of CLV—a peptide which encompasses a large sequence of the cleavage region of the Env precursor—delayed, but did not prevent, gp160 processing, whereas it fully abolished syncytium formation. This compound is more potent than the PCMKs previously described (Hallenberger *et al.,* 1992). The ability of a labeled CLV analog to enter cells is in agreement with such an intracellular effect. However, this interference with gp160 cleavage was not sufficient to alter substantially the amount of cleaved Env produced and expressed at the cell surface. The observed shedding into the cell supernatant of mature cleaved gp120 is another evidence that gp160 cleavage was not blocked by the treatment by CLV. Surprisingly, the presence of CLV slightly increased Env membrane expression, in agreement with the previous observation of Hallenberger *et al.* (1992), who observed that PCMKs slightly decrease shedding of gp120.

Because CLV altered at least the cleavage process during biosynthesis, we hypothesized that the presence of a cleavage substrate sequence on CLV was actually responsible for its interference with membrane fusion. To address this point, we synthesized various analogs of CLV on the basis of the data obtained in a previous study based on site-directed mutagenesis, which determined the aa required for Env cleavage (Bosch and Pawlita, 1990; Einfeld, 1996). First, we synthesized a CLV analog which presents a R > S substitution in the cleavage sites. Such a mutation on *env* impairs Env cleavage (Bosch and Pawlita, 1990). Such an analog was inactive. Similarly, an analog which presents a REKR motif close to the uncharged Lys core lacked activity (The Lys core is uncharged as a result of the SPC synthesis). In full contrast, the M analog which presents a functional site 1 was active, despite the removal of several aa upstream to the functional cleavage site. From these data, we conclude that the sole presence of a functional cleavage region is responsible for CLV activity. This further argues in favor of a CLV-induced inhibition of syncytium formation through interferences with the enzymatic machinery involved in gp160 processing. Additional interferences of CLV with some steps of the membrane fusion process per se may act together with the interference with Env cleavage to completely impair membrane fusion. It is also possible that the presence of both cleaved and uncleaved Env within the same oligomers late in the intracellular routing or at the membrane disturbs the Env functions and structure to inactivate it. These hypotheses are under investigation to explain why CLV exhibits so potent an effect on Env functions despite its apparently slight effect on Env processing.

MATERIALS AND METHODS

Materials

D7324 (Aalto, Dublin, Ireland) is a polyclonal antibody (Ab) against gp120 Ct (aa 502–516); D7323 is against gp41 Ct (aa 845–860); 9305 and 9301 (DuPont de Nemours, Dreieich, Germany) are mouse monoclonal anti-gp120 Abs directed against aa 318–328 and aa 475–486, respectively. Chemical products were of analytical

grade (Sigma, St Louis, MO). Products for peptide synthesis were from Perkin–Elmer (Paris, France).

Synthesis of synthetic polymeric constructions

Chemical synthesis of SPCs was performed by the solid phase technique using Fmoc/t-butyl chemistry, as described by Yahi *et al.* (1994). The crude peptides were purified to homogeneity by C18 reversed-phase medium-pressure liquid chromatography (Barbouche *et al.*, 1998a,b). The purity of the peptides was assessed by analytical C18 reversed-phase high-pressure liquid chromatography (Barbouche *et al.*, 1998a,b). Fractions containing purified peptides were pooled and characterized by both aa analysis after acid hydrolysis and mass spectrometry. They were then tested in bioassays. SPCs are as follows: SPC C (a negative control): ($[GPGKTL]_8$-K_4-K_2-K-βA); SPC CLV: ($[KIEPLGVAPTKAKRRVVQREKR]_4$-$K_2$-K-$\beta$A); SPC CLV L (long): ($[KIEPLGVAPTKAKR\underline{S}VVQREK\underline{S}]_4$-$K_2$-K-$\beta$A); SPC CLV M (medium): ($[PTKAKRRVVQREKR]_4$-K_2-K-βA); SPC CLV S (short): ($[VVQREKR]_4$-K_2-K-βA); and SPC CLV Y (CLV analog used for radiolabeling): ($[PTKAKRRVVQREKR]_4$-K_2-K-Y).

Syncytium formation

Human lymphoid $CD4^+$ CEM cells—cultured in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS) and 1% Gln—were infected in 12-well plates in the presence of various SPCs for 18 h with a recombinant vaccinia virus (rVV) expressing HIVLai Env (Kiény *et al.,* 1988; VV9-1; 0.2 PFU/cell; a gift of Marie-Paule Kièny, Transgène, Strasbourg, F), as described by Fenouillet *et al.* (1993).

Env production and quantitation

Baby hamster kidney (BHK-21) cells—cultured in Glasgow medium supplemented with 5% FCS and 1% Gln—or CEM cells were infected as described above. Infections were performed in FCS-free medium to allow, after 1 or 2 days, supernatant concentration [5-fold using a 30-kDa cutoff Centriprep system (Amicon, Danvers, MA)] (Fenouillet *et al.,* 1993); cell pellets were lysed in phosphate-buffered saline, pH 7.4 (PBS), 0.1% Triton X100.

The quantities of Env obtained in the different systems were determined by dot blot (described in Fenouillet and Gluckman, 1991) and ELISA (Fenouillet *et al.,* 1993), using as reference a cell lysate spiked with a known amount of recombinant Env (Fenouillet *et al.,* 1992, 1993, 1996). Briefly: (i) dot blot: 5, 2.5, and 1 μl of lysate (10^6 cells/10 μL of lysate) or 5× supernatant (10^6 cells/100 μL) adjusted to 10 μl final volume of PBS were blotted onto a nitrocellulose filter. Staining was performed with a pool of HIV^+ sera (1:50) which was devoid of detectable Abs against VV (Fenouillet *et al.,* 1993) and preincubated with CLV ($3 \cdot 10^{-4}$ M) to inhibit a possible bias induced by the reactivity of Abs with CLV present in the samples. Under these conditions, the Abs used as described below reacted neither with uninfected cells incubated with 10 μM CLV nor with the medium or cell lysate. Peroxidase-labeled anti-human IgG Abs (Dakopatts, Glostrup, Denmark) were then added (1/100). (ii) ELISA: wells (Nunc, Roskild, Denmark) were coated (200 ng/100 μL) with D7324—to detect gp160 and gp120—or D7323—to detect gp160 and gp41. Lysates or 5× supernatants were added. After washing, the pool of HIV^+ sera was added and staining was performed as described above.

Alternatively, a Western blot analysis of Env present either in cell supernatant or in cell lysate was performed as reported by Fenouillet *et al.* (1996). Briefly, concentrated (5×) supernatants (50 μL) and cell lysates ($2 \cdot 10^6$ cells/50 μL) were submitted to SDS–polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions. After blotting, staining was obtained (i) with human anti-HIV-1 Abs (diluted 1/100 to detect Env species in the supernatant), (ii) with a mixture of 9305 and 9301 mAbs (diluted 1/50 to detect gp120 and gp160 in the lysates), or (iii) with D7323 (diluted 1/100 to detect gp41 and gp160 in the lysates). Incubations and washes were performed in PBS 0.5% casein, 0.5% Tween 20. Peroxydase-conjugated anti-Abs were then incubated for 1 h before staining with diaminobenzidine.

Detection of Env membrane expression

Cells were infected with rVV for 1 or 2 days. After a wash in PBS, cells were treated with 2% paraformaldehyde. After 18 h, $3 \cdot 10^6$ cells were washed in PBS 2% casein (PBSC) and incubated for 2 h with a saturating concentration (1:100) of the pool of HIV^+ sera to detect membrane Env as described (Fenouillet *et al.,* 1996, 1997). After a wash, peroxidase-coupled anti-Abs were added (1/50 in PBSC) for 1 h. After washes, chromogen was added. The labeling was specific for the membrane expression of Env, as it was dependent on the Ab concentration and as incubation with a pool of HIV^- sera did not label infected cells (Fenouillet *et al.,* 1997, and data obtained under these conditions and not shown).

Env precursor cleavage

gp160 cleavage into gp120 and gp41 was examined by pulse–chase labeling as described in Fenouillet *et al.* (1995, 1997). Briefly, after starvation 15 h postinfection for 1 h, 10^7 cells were labeled for 30 mn with a [^{35}S]Met/Cys mixture (Amersham, UK; 300 mCi); Met-supplemented (100 μg/mL) medium was then added for 30 or 120 min. Cells were lysed in Tris 25 mM, 300 mM NaCl, pH 7.4, 0.5% Triton X-100, and 0.25% SDS and a pool of HIV^+ sera (1:50) was added for 3 h in the same buffer. Protein A–Sepharose (Pharmacia, Uppsala, Sweden) was then added. After washes in the lysis buffer, elution was

obtained with 10% SDS, 10% β-mercaptoethanol before analysis by 10% SDS–PAGE (Fenouillet *et al.,* 1997).

Internalization of an active radiolabeled CLV analog

The active CLV Y (5 μg) was labeled with ^{125}I-labeled Na (300 μCi) in a 4 nM iodogen-coated tube for 30 mn at 20°C (Barbouche *et al.,* 1998a,b). Labeled CLV Y (sp act 50 μCi/μg) was purified on a PD10 column (Pharmacia). CEM cells were incubated with ^{125}I-labeled CLV Y in cell culture medium for various times. Triplicates were performed ($n = 2$ experiments). For each incubation time, a triplicate was similarly incubated but the presence of 0.1% NaN_3 to avoid endocytosis as reported in Barbouche *et al.* (1998b). Samples were then washed twice with PBSC, 0.1% NaN_3 and cell-associated radioactivity was counted. Cell-associated radioactivity when incubation was performed in the presence of NaN_3 represented membrane-associated CLV Y. Accordingly, ^{125}I-labeled CLV Y present within the cells was calculated for each time point as follows: "cell-associated radioactivity obtained when incubation was performed in the absence of NaN_3" minus "cell-associated radioactivity obtained when incubation was performed in the presence of NaN_3," as reported by Barbouche *et al.* (1998b).

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